

THESIS

MANAGEMENT OPTIONS FOR THE WALNUT TWIG BEETLE, *PITYOPHTHORUS*
JUGLANDIS BLACKMAN, VECTOR OF THE FUNGAL CANKER PATHOGEN
GEOSMITHIA MORBIDA

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ABSTRACT

MANAGEMENT OPTIONS FOR THE WALNUT TWIG BEETLE, *PITYOPHTHORUS* *JUGLANDIS* BLACKMAN, VECTOR OF THE FUNGAL CANKER PATHOGEN *GEOSMITHIA MORBIDA*

The walnut twig beetle, *Pityophthorus juglandis* (Blackman) and its fungal associate *Geosmithia morbida* have recently been discovered to produce thousand cankers disease. *Pityophthorus juglandis* is native to the southwestern United States, but its geographic range is expanding, as is incidence of thousand cankers. The disease occurs in multiple walnut species, but it has devastated black walnut (*Juglans nigra*) populations in several western states. To better understand and manage thousand cankers disease, studies were initiated to (1) determine monthly variations in *P. juglandis* cold tolerance, (2) develop effective management tools for infested felled logs, and (3) determine suitable fungicides to treat thousand canker diseased trees and techniques to create *Geosmithia*-free beetle populations.

Monthly variations in cold tolerance were measured by the supercooling point (SCP) to predict the survival of *P. juglandis* within the native range of black walnut. Mean SCPs were the highest for larvae (-14.2°C) in the month of September and lowest for adults in October (-19.7°C). These studies, as well as beetle survival in infested Colorado trees where temperatures reached -29°C in February 2011, suggest *P. juglandis* could survive the winter in much of the native range of black walnut, but may be limited in trees where temperatures regularly exceed the lower lethal temperatures.

Finding effective management options for thousand cankers disease is critical to decrease further expansion into urban and eastern walnut forests by the movement of infested walnut

wood. In a series of experiments, we tested the effect of insecticide, temperature, submergence, and chipping treatments on *P. juglandis*. Insecticidal treatments were more than 80 percent effective. Heat treatments of 60°C for 30 minutes and cold treatments of -25°C for seven days were effective phytosanitary methods to control *P. juglandis*. Although temperature treatments eliminated *P. juglandis* populations, re-colonization occurred if logs were exposed to beetles. Soaking logs for eight days in 70% ethanol sanitized logs, but *P. juglandis* survived water soaks. Chipping infested material was over 90 percent effective, and emergence from chipped material ceased after two weeks. These experiments provide baseline management options for walnut logs infested with *P. juglandis*, and show the importance of properly storing treated logs to prevent re-colonization.

Suitable fungicides were determined for their ability to inhibit or kill in-vitro *Geosmithia morbida* colonies. In an attempt to obtain sterile beetles, fungicide, bleach, and ethanol rinses were tested. Ten µg/g of thiophanate-methyl (active ingredient in Cleary's 3336^F) inhibited *G. morbida* growth by 20%, but the fungus was inhibited by greater than 97 percent in potato dextrose agar amended with 10 µg/g or higher concentrations of the fungicide azoxystrobin (active ingredient in the fungicide Heritage). In contrast the fungus was completely inhibited and killed in agar amended with 0.6 µg/g azoxystrobin and 1.0 µg/g propiconazole (active ingredients in the fungicide Headway) along with 6.4 µg/g of propiconazole (active ingredient in the fungicide Banner MAXX). Using a first order decay regression model, the effective concentrations needed to decrease *G. morbida* mycelial growth by 50 percent and 90 percent (EC₅₀ and EC₉₀ respectively) were determined for the active ingredients azoxystrobin and/or propiconazole. The combination of sterilizing agents used in this experiment did not effectively disinfest *P. juglandis* adults or larvae of *G. morbida*.

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CHAPTER I. The Effect of Cold Temperature on Survival of *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae)¹

Preface:

The information presented in this first chapter, in combination with data collected by Emily Luna, has been accepted in the *Journal of Environmental Entomology*. The article by Emily Luna, Rachael Sitz, Whitney Cranshaw, and Ned Tisserat is entitled “The effect of temperature on survival of *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae).” I collected all of the results and co-wrote the information included in this chapter.

INTRODUCTION

The walnut twig beetle, *Pityophthorus juglandis* Blackman, is a bark beetle native to *Juglans* species that occur in the southwestern United States and Mexico (Blackman 1928, Bright and Stark 1973). The beetle develops in the phloem of small twigs or larger, damaged branches in these species. It is also commonly, if not invariably, associated with *Geosmithia morbida*, a fungus that colonizes and kills tissues surrounding beetle-produced feeding wounds and galleries (Tisserat et al. 2009, Kolarik et al. 2011). In resistant hosts (e.g., *Juglans major*) the resulting cankers are quickly localized in the bark by wound periderm formation and remain small and superficial (Utley et al. 2013).

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Over the past couple of decades, *P. juglandis* has greatly expanded its geographic range within North America (Cranshaw 2011, Seybold et al. 2012, Utley et al. 2013). This has caused the insect to become associated with new hosts, notably *Juglans nigra* (black walnut), a species native to eastern North America but widely planted in the western United States (Tisserat et al. 2009). Both *P. juglandis* and *G. morbida* are far more aggressive in these non-native hosts (Utley et al. 2013). In western states *P. juglandis* can produce enormous populations in black walnut trees where breeding may also involve large diameter branches and the trunk.

Geosmithia morbida produces diffuse cankers that may coalesce and eventually kill the bark in susceptible hosts. This combined damage, given the name thousand cankers disease, has resulted in widespread mortality of *J. nigra* throughout the western United States (Tisserat et al. 2009, Tisserat et al. 2011). More recently thousand cankers disease was detected within the native range of *J. nigra*, being present in areas of Tennessee, Virginia, Pennsylvania, and North Carolina (Grant et al. 2011, VDA 2011, PDA 2011, NCSU 2013).

Within the broad geographic area where *P. juglandis* and *G. morbida* are now present wide ranges in temperature exist, and temperature may have several effects on the causal organisms associated with thousand cankers disease. The number of generations in subcortical insects, such as *P. juglandis*, is influenced by temperature (Graham 1924, Lombardero et al. 2000) and shorter generation times are expected with warmer temperatures. Conversely, lower lethal temperatures potentially limit the survival of the insect in areas where extreme cold temperatures occur. This has been demonstrated for the southern pine beetle, *Dendroctonus frontalis* Zimmerman, whose northern distribution limits change based on winter temperature extremes (Ungerer et al. 1999). Lower lethal temperature limits for *P. juglandis* have not been previously reported.

Insects exposed to low temperatures are threatened by intracellular ice formation (Denlinger and Lee 1998). A commonly accepted hypothesis on freezing separates insects into two categories: freeze-tolerant species or freeze-susceptible species (Salt 1961, Lee and Denlinger 1991, Bale 2002). Freeze-tolerant insects are adapted to protect their cells from damage during the freezing process. On the contrary, freeze-susceptible insects avoid freezing by overwintering in protected habitats, using cryoprotectant substances such as glycerol to lower the point of ice nucleation (Lee and Denlinger 1991), and clearing their gut content of food materials which could act as ice nucleators (Bale 2002). In freeze-susceptible insects, the supercooling point (SCP), defined as the temperature at which spontaneous freezing occurs (Régnière and Bentz 2007), is usually the same as the absolute lower lethal temperature (LT) threshold (Lee and Denlinger 1991).

To better understand low temperature effects on survival of *P. juglandis*, studies were conducted to determine monthly variations in cold tolerance as measured by the supercooling point.

MATERIALS AND METHODS

Supercooling Point Determination. Black walnut trees showing symptoms of thousand cankers disease were felled in urban areas adjacent to the front range of Colorado in 2012. *Pityophthorus juglandis* infested logs were stored in 66 liter clear plastic tubs in a shaded screened facility at Colorado State University. Beetle extractions and supercooling point experiments took place from June 2012 through January 2013.

Adult and larval walnut twig beetles were found by peeling back layers of cambium tissue with a scalpel, and then removed with a wet fine bristled paintbrush. Male and female adult *P. juglandis* were sexed according to taxonomic descriptions outlined by Blackman (1928).

Once beetles were successfully excised and sexed, they were transferred to a 1.0 mL plastic tube and stored at 25°C for 12 to 36 hours prior to each experiment.

To determine monthly supercooling points, *P. juglandis* were fixed in an environmental chamber where they were exposed to decreasing temperatures. At least twelve adults and larvae were collected to test each month (Tables 1.1 and 1.2), and at least eight specimens of each gender were tested each month starting with September 2012, and through January 2013 (Table 1.3). To begin, beetles were transferred to the temperature chamber with a fine bristled paintbrush coated with high vacuum grease (Dow Corning, Midland, MI). The grease held each beetle in place between a 36-gauge copper-constantan thermocouple and an aluminum rod used to track temperature changes. They were placed into an aluminum block designed for temperature stabilization within an environmental test chamber programmable freezer (Tenny Jr. Programmable Freezer, Tenny, Inc., South Brunswick, N.J.).

Initially, the temperature in the chamber was set at 25°C. In order to immobilize the beetles, the temperature quickly dropped to 0°C for two minutes (Carrillo et al. 2005) then temperatures were lowered at a rate of -0.2°C per minute (Lombardero 2000) until it reached -25°C. A multi-channel data logger (DaqPro-5300, Portable Handheld Data Logger, Omega Engineering, Inc., Stamford, CT.) recorded thermocouple temperatures every second. SCPs were determined by visualizing a peak with a rise in temperature greater than 0.5°C. The lowest temperature recorded before the peak specified the onset of freezing. *Pityophthorus juglandis* mortality was checked within 24 hours of the experiment, and beetle survival was determined by movement after aggravation with a fine-bristled paint brush.

Statistical Analysis. Differences between all monthly SCP values (adults, larvae, male, and female values) were detected using the Wilcoxon method to account for non-normality and

unequal variances (SAS 2012, JMP Statistical Software, version 10). The statistical program R was used to graph monthly SCP values for adult and larvae *P. juglandis* along with male and female *P. juglandis* (R Development Core Team, Vienna, Australia).

RESULTS AND DISCUSSION

Effect of Month on SCP. Differences were detected in adult monthly supercooling points. Adult *P. juglandis* monthly mean supercooling points ranged between -14.4°C in July to -19.7°C in October (Table 1.1). Adult supercooling points were higher in July and August than in June ($P = 0.0008$ and $P = 0.0004$ respectively) and January ($P = 0.0001$ and $P < 0.0001$ respectively). Supercooling points were lower in November and December when compared to values in July ($P < 0.0001$ for both months) and August ($P < 0.0001$ for both months), but higher than October values ($P = 0.0010$ and $P = 0.0019$ respectively). September adult supercooling points were lower than July and August ($P = 0.0061$ and $P = 0.0059$ respectively), and October values were lower than all months other than November and December (June $P = 0.0292$; July $P < 0.0001$; August $P < 0.0001$; September $P = 0.0001$; and January $P < 0.0001$). Overall, *P. juglandis* adults lowered their supercooling point values in the months leading to winter, and in winter months.

Table 1.1. Mean (\pm SE) and range for adult *Pityophthorus juglandis* monthly supercooling points.

2012/2013 Month	<i>n</i> ^a	Mean SCP (°C) \pm SE	Range (°C)
June	16	-18.0 \pm 0.60	-13.0, -21.8
July	13	-14.4 \pm 0.65	-8.6, -19.4
August	19	-14.8 \pm 0.40	-11.2, -18.1
September	18	-16.8 \pm 0.49	-13.2, -20.0
October	17	-19.7 \pm 0.35	-16.1, -22.4
November	21	-17.8 \pm 0.44	-15.2, -22.9
December	31	-17.7 \pm 0.40	-14.9, -22.8
January	25	-17.3 \pm 0.42	-12.1, -23.4

^aNumber of adults tested each month.

Larval monthly mean supercooling point differences were also identified, and ranged from -14.2°C in September to -17.8°C in November (Table 1.2). July, August, and September supercooling point values were higher than values in June ($P = 0.0002$; $P = 0.0129$; and $P = 0.0008$ respectively) and January ($P = 0.0010$; $P = 0.0194$; and $P = 0.0009$ respectively). In contrast, values in October, November, and December were lower than in July ($P = 0.0009$; $P = 0.0002$; and $P < 0.0001$ respectively), August ($P = 0.0052$; $P = 0.0016$; and $P = 0.0006$ respectively), and September ($P = 0.0008$; $P = 0.0002$; and $P < 0.0001$ respectively). A separation in larval monthly supercooling point values is seen between July, August, and September when compared to October, November, and December. Mean monthly larval supercooling points lowered in response to colder weather.

Table 1.2. Mean (\pm SE) and range for larval *Pityophthorus juglandis* monthly supercooling points.

2012/2013 Month	<i>n</i> ^a	Mean SCP (°C) \pm SE	Range (°C)
June	12	-16.5 \pm 0.34	-14.9, -18.2
July	12	-14.3 \pm 0.32	-11.8, -16.5
August	16	-15.3 \pm 0.34	-12.8, -17.6
September	18	-14.2 \pm 0.52	-8.3, -18.8
October	18	-17.5 \pm 0.69	-12.1, -22.3
November	17	-17.8 \pm 0.61	-13.8, -22.3
December	16	-17.3 \pm 0.27	-15.9, 19.3
January	15	-16.5 \pm 0.56	-10.3, -19.9

^aNumber of larvae tested each month.

Seasonal variation in supercooling points for an insect species is not uncommon (Carrillo and Cannon 2005, Lombardero et al. 2000, Renault et al. 2002), and we observed that *P. juglandis* excised from the same log showed monthly mean variations of about 5°C in adults and 3°C in larvae. Seasonal fluctuations in supercooling point have been documented in many insects (Bentz and Mullins 1999), though the extent of the fluctuation and the temperature tolerances of species vary (Denlinger and Lee 1998). For example, *P. juglandis* exhibits higher monthly changes in supercooling point when compared to *Cerotoma trifurcate* (Forster) bean leaf beetles (Carrillo et al. 2005). When compared to other insects, the Indianmeal moth, *Plodia interpunctella* (Hübner), decreased its SCP by more than 10°C (Carrillo and Cannon, 2005) and the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, by more than 15°C (Bentz and Mullins, 1999). Although differences in *P. juglandis* SCP's were detected between seasons, their magnitude was not as extreme as noted in some other insect species. This might be explained by the fact that *P. juglandis* is not exposed to extremely cold conditions in its native range and therefore has no need for extreme supercooling during winter.

Effect of Stage on SCP. Supercooling point differences between adults and larval beetles were detected in September and October 2012 ($P = 0.002$ and $P = 0.016$ respectively). Adults were slightly more cold tolerant than larvae in September and October, but all other months were not different from each other (Figure 1.1). Stage specific effects on supercooling point have been documented. For example, larvae of the multicolored Asian lady beetle *Harmonia axyridis* (Pallas) have supercooling points a few degrees lower than adults (Koch et al. 2004). Larval instars vary in feeding habits and size, which are attributes that influence cold tolerance (Denlinger and Lee 1998). Bentz and Mullins (1999) outlined the research conducted on cold tolerance for each stage of the mountain pine beetle, *Dendrocronus ponderosae*; large larvae were the most cold tolerant. On the contrary, fifth instar *P. interpunctella* larvae were less cold hardy than first instar larvae and adults (Carrillo and Cannon 2005). In this study, later instar larvae were used more frequently due to their larger size. We do not know whether or not grouping *P. juglandis* larval instars together influences the mean supercooling point.

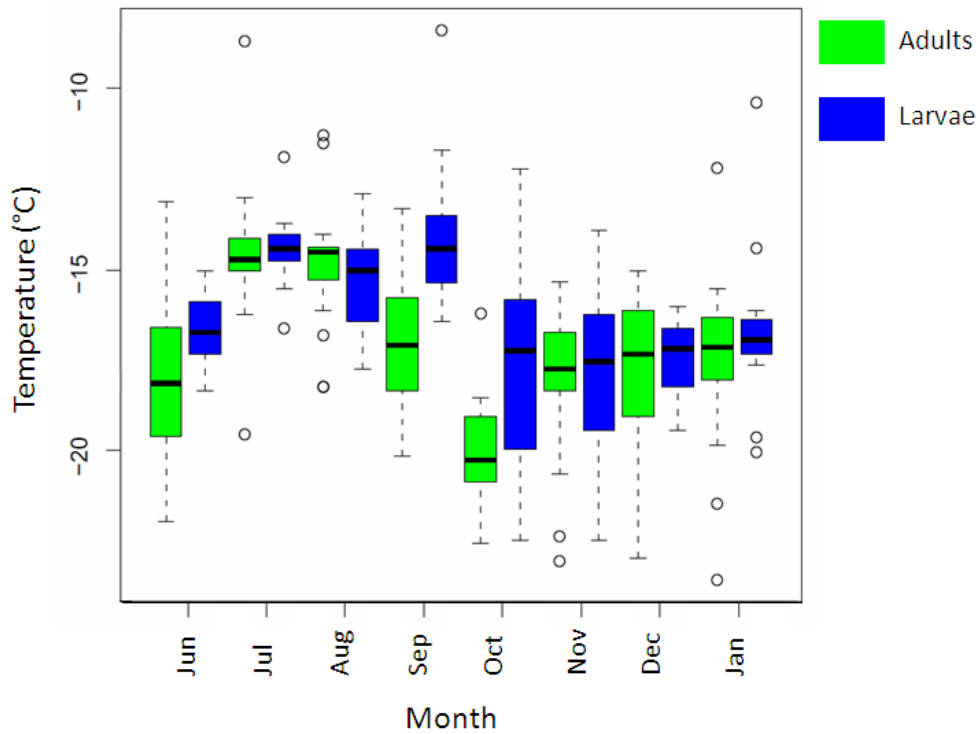


Figure 1.1. Variations in Adult and Larval Monthly Supercooling Points. Supercooling points for *Pityophthorus juglandis* adults (green) and larvae (blue) are shown from June 2012 through January 2013. The upper and lower ends of each box represent the 25th and 75th percentiles. The sample median is indicated by the central horizontal line in each box. Maximum and minimum values are represented by horizontal line at the top and bottom end of the whiskers, and circles indicate outliers.

Effect of Gender on SCP. *Pityophthorus juglandis* adult supercooling points were tracked according to gender from September 2012 to January 2013 in order to account for monthly SCP variation (Figure 1.2). Mean supercooling point values ranged 4°C in males, while females ranged 3°C (Table 1.3). In some species, sexual dimorphism can result in SCP differences (Renault et al. 2002), but monthly gender differences were not detected in *P. juglandis* ($P = 0.706$). Therefore it does not appear that sexual dimorphism contributes to significant differences in SCP of *P. juglandis*.

Table 1.3. Mean (\pm SE) and range of monthly supercooling points for male and female adult *Pityophthorus juglandis*.

2012/2013 Month	<i>n</i> ^a	Mean SCP (°C) \pm SE	Range (°C)
Male <i>P. juglandis</i>			
September	9	-15.8 \pm 0.53	-13.2, -19.5
October	9	-20.1 \pm 0.47	-18.4, -22.4
November	10	-18.2 \pm 0.79	-15.4, -22.9
December	15	-17.5 \pm 0.44	-15.2, -21.3
January	10	-18.1 \pm 0.81	-15.4, -23.4
Female <i>P. juglandis</i>			
September	9	-17.8 \pm 0.51	-15.3, -20.0
October	8	-19.4 \pm 0.54	-16.1, -20.7
November	11	-17.4 \pm 0.45	-15.2, -20.5
December	15	-17.8 \pm 0.69	-14.9, -22.8
January	15	-16.7 \pm 0.41	-12.1, -18.3

^aNumber of beetles tested each month.

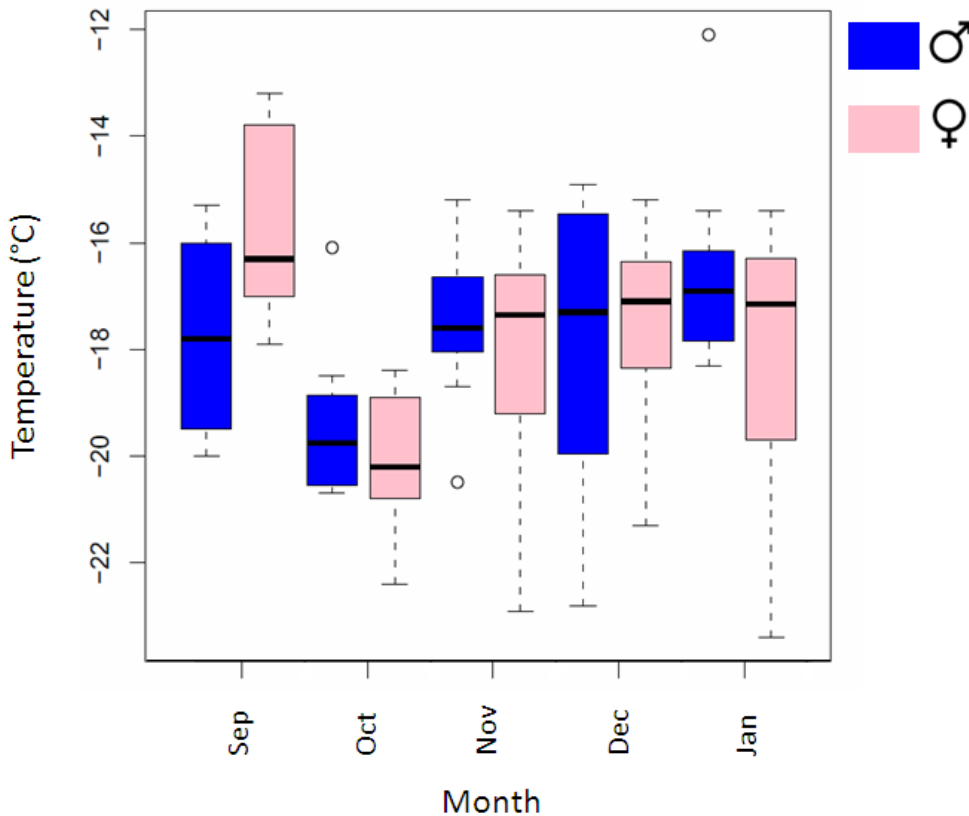


Figure 1.2. Variations in Adult Male and Female Monthly Supercooling Points. Supercooling points for male (blue) and female (pink) adult *Pityophthorus juglandis* are shown from September 2012 to January 2013. The upper and lower ends of each box represent the 25th and 75th percentiles. The sample median is indicated by the central horizontal line in each box. Maximum and minimum values are represented by horizontal line at the top and bottom end of the whiskers, and circles indicate outliers.

Effect of Environmental and Physiological Acclimation on SCP. *Pityophthorus*

juglandis survival may be limited by extreme cold events in the northern native range of *J. nigra*, where temperatures of -18°C or lower, and below the SCP of the insect, are not uncommon.

Nevertheless, it is worthy of note that *P. juglandis* adults and larvae were observed to survive within infested trees following a brief exposure to -25°C in February 2011 in Lyons, Colorado.

This suggests that bark may play a role in insulating *P. juglandis* from short durations of extremely low temperatures and such effects are likely more pronounced when insects are found within sites of thick bark (Régnière and Bentz 2007). Branches of *J. nigra* have thinner bark

when compared to the thick bark on the trunk and in early stages of thousand cankers disease beetle infestations are concentrated in limbs (Tisserat et al. 2009).

Gradually decreasing temperatures induce physiological changes in *D. ponderosae* (Bentz and Mullins 1999), and their survival is directly correlated with time and temperature (Yuill 1941, Bentz et al. 1991, Safranyik and Linton 1998). Larval populations of *D. ponderosae* experienced eighty percent mortality when exposed to temperatures around their lowest supercooling point of -34.4°C for 32 days (Safranyik and Linton 1998). However, if unseasonably low temperatures occur before cold hardening they can cause increased mortality (Safranyik and Linton 1991). For example, cold periods of -26°C that occurred early in the season decreased the overwintering survival of *D. ponderosae* (Safranyik and Linton 1991). Similar to *D. ponderosae*, low temperatures may not need to reach established supercooling points to cause mortality in *P. juglandis* populations. Further studies are needed, more specifically, exploring the effects of freezing temperatures and their durations on *P. juglandis*.

Although the life cycle of *P. juglandis* in Colorado is incompletely understood, their gut may be cleared of ice nucleating agents associated with freezing, which in turn lowers the SCP. This seasonal dichotomy of SCPs attributed to food particles acting as ice nucleators has been identified in other arthropods (Worland and Convey 2001, Régnière and Bentz, 2007). Some members in the subfamily Scolytinae produce cryoprotectants including polyhydric alcohols and antifreeze proteins (Lee and Denlinger 1991, Duman 2001). Further studies are needed to determine if *P. juglandis* similarly produce these cryoprotectants.

Better understanding of the effects of temperature on the survival of *P. juglandis* can be a key factor in understanding its population dynamics and can also help to develop effective pest management techniques. The results of this study provide a baseline of lower lethal temperatures

that can be useful in predicting the survival and development of *P. juglandis* when host plants are exposed to temperature extremes. Determination of lethal temperatures also can provide guidance toward development of treatments to disinfest wood of *P. juglandis*.

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CHAPTER II. Sanitation Measures to Control Walnut Twig Beetle (Coleoptera: Curculionidae)

Emergence from Felled Black Walnut Logs

INTRODUCTION

Black walnut, *Juglans nigra*, is a highly valued tree (Harlow and Harrar 1969) with wood used for furniture, gunstocks, and veneer (Newton et al. 2009). In the eastern United States lumber industry, *J. nigra* growing stock is estimated at half a trillion dollars (US) and annually produces exports valued at 325 million dollars (US) (Newton et al. 2009). It is a highly valued wood, producing veneer quality boards worth between \$800 and \$6,000 per thousand board feet. *Juglans nigra* is native to the eastern United States, but it is more widely planted, primarily as a landscape tree (Williams 1990). Standing trees are an important source of food for wildlife, and many communities have rich histories harvesting the nut crop.

In recent years, healthy *J. nigra* have been killed by mass colonization of the walnut twig beetle, *Pityophthorus juglandis* (Blackman), and the pathogenic fungal associate *Geosmithia morbida* (Tisserat et al. 2009). *Geosmithia morbida* creates cankers around beetle galleries or other tree wounds produced by the beetle (Tisserat et al. 2009, Leslie et al. 2010). The cumulative effects of beetle wounding and canker development are known as thousand cankers disease (TCD). The disease is manifested as flagging and dieback in the upper tree canopy (Tisserat et al. 2009). In susceptible hosts (e.g., *J. nigra*) the disease often progresses to produce tree mortality (Utley et al. 2013).

A native host of *P. juglandis* from which it was originally described is *J. major* (Arizona walnut), located in the southwestern United States and northern Mexico (Blackman 1928, Cranshaw 2011). Records during the 1950's also show colonization of *J. californica* (Southern

California walnut) in California (Bright and Stark 1973), and this species is also generally accepted as a native host of the beetle.

Thousand cankers disease was first identified in western states (Arizona, California, Colorado, Idaho, Nevada, Oregon, Utah, and Washington), but was more recently found in several pockets of the eastern United States (North Carolina, Pennsylvania, Tennessee, and Virginia) (Cranshaw 2011, Grant et al. 2011, Kolarik et al. 2011, Seybold et al. 2012a, North Carolina Department of Agriculture and Consumer Services 2013, Tisserat et al. 2009). *Juglans nigra* losses have been particularly extensive in Rocky Mountain States where thousand cankers disease has largely extirpated the species from many communities where it had been planted (Tisserat et al. 2011).

Finding an effective control of thousand cankers disease is critical to the protection of both urban and native forest stands of susceptible *Juglans* species. Present local programs emphasize the importance of removing contaminated material to slow the spread of thousand cankers disease (Haun et al. 2010), but the value of black walnut wood complicates the removal process. Logs are frequently salvaged from thousand cankers disease affected trees, and the contaminated logs remain highly infectious (Newton et al. 2009). For this reason, identifying methods to safely handle contaminated wood are needed to eliminate the risk of spreading thousand cankers disease.

There are many kinds of treatments used to manage bark beetle and borer pests (e.g. Coleoptera: Buprestidae, Cerambycidae, and Curculionidae) including: heat treatment, kiln-drying, fumigation, insecticidal sprays, solarization, debarking, and insect behavior modification chemicals (Nzokou et al. 2008, FAO 2002, Hastings et al. 2001, Negron et al. 2001, Jones et al. 2013, Seybold et al. 2012b). However, to date, effective controls have not been well developed

for *P. juglandis*.

The following studies describe investigations of several methods for their ability to disinfest *P. juglandis* infected wood and properly store logs before transport. Also recorded were the effects of these management strategies on other non-target insects associated with *J. nigra*.

MATERIALS AND METHODS

Host Plant Material. Two trees showing advanced symptoms of thousand cankers disease were felled in Fort Collins, Colorado on 19 July 2012. Logs salvaged from these trees were subsequently used in a series of studies, all initiated within a few weeks of harvest. Treatments were applied to whole logs. Each replication consisted of logs cut from a section of the tree into 30.5 cm lengths with an average diameter of 11.6 cm. The experimental design was a randomized complete block. To prevent desiccation, cut ends of logs were painted with a paraffin-based wax (Anchorseal®, U.C. Coatings Corporation, Buffalo, NY). Logs were held in 66-liter clear plastic tubs with lids amended with breathable fabric and placed in a shaded screen facility on the Colorado State University in Fort Collins for later use in trials.

Emergence and Collection Boxes. Each log was placed in an emergence box and kept inside of a screen house exposed to ambient outside temperatures. Emergence containers, modified from Peachey (2012), were constructed from cardboard boxes (61 x 25 x 25 cm, ULINE, Pleasant Prairie, WI) amended to include 50 mL clear plastic tubes screwed into threaded 90° polyvinyl chloride (PVC) elbows. PVC surfaces were sanded to provide friction for the walnut twig beetles to travel into the tubes. All of the box seams were sealed with hot-glue, and duct tape was applied over the top to avoid light penetration. Adult *P. juglandis*

emerged from log sections into the PVC tubes naturally due to positive phototaxis. Weekly emergence for all arthropod specimens was collected and quantified until 5 November 2012.

In addition to *P. juglandis*, several other species emerged from the stored logs. The genera of particular abundance included *Enoclerus* (Coleoptera: Cleridae), *Corticotomus* (Coleoptera: Trogossitidae), *Theocolax* (Hymenoptera: Pteromalidae) and *Cryptolestes* (Coleoptera: Latridiidae) (Appendix I, Table A.1.1). All arthropods emerging from logs were collected and recorded (Appendix I, Table A.1.2).

Insecticide Trial. Currently, bark beetle management programs often utilize carbaryl, permethrin, and/or bifenthrin insecticides to control infestations or remediate attacked standing trees (Grosman et al. 2010, Grosman and Upton 2006). Based on preliminary results, two insecticides, carbaryl (Garden Tech Sevin Concentrate Bug Killer, 22.5% carbaryl) and bifenthrin (Onyx, 23.4% bifenthrin), were evaluated as drenching sprays for their ability to prevent *P. juglandis* emergence. The experimental design contained three treatments (carbaryl, bifenthrin, and water check) with 10 replications. Treatments were applied on 20 July as a drenching spray until runoff to all surfaces. Rates of carbaryl and bifenthrin were based on high-end rates of applications labeled for bark beetles: carbaryl was applied at 1:100 dilution, bifenthrin at 1:400 dilution. Upon drying, all ends of logs were sealed with Anchorseal and placed in emergence boxes on 23 July.

Heat Trial. Although high lethal temperatures are unlikely in natural settings, heat treatments are commonly used to sanitize wood packing material (ISPM-15, FAO 2002) and felled landscape trees of insect pests (Nzokou et al. 2008; Mushrow et al. 2004; Myers and Bailey 2011). Heat treatment efficacy was tested on *P. juglandis* by exceeding the international standards for phytosanitary guidelines for wood packing material (ISPM-15, FAO 2002) and

followed recommendations set by Costanzo (2012), ensuring the cut walnut logs reached at least 56°C for 30 minutes.

In order to determine if heat-treated phloem tissue remains a suitable feeding or reproductive environment for *P. juglandis*, half of the heated logs were re-exposed to adult beetles. Logs were arbitrarily assigned to one of the three treatments (untreated check, heat treated, and heat treated with beetle re-infestation), each with 10 replications. Logs were sealed with Anchorseal on 20 July, heat-treated on 23 or 24 July, and then placed in emergence boxes.

Heat treatments were performed by placing logs on the top shelf of a 220 volt 12 kw drying oven (Despatch Oven Co. Minneapolis, MN). In order to obtain heartwood temperatures, data loggers that recorded temperatures every minute (WatchDog Model 450, Spectrum Technologies, Inc. Plainfield, IL) were inserted into holes drilled in the center of the logs at a depth of 38 mm and diameter 9.5 mm, perpendicular to the wood grain. We ensured that temperatures below the bark reached between 60°C and 72°C for 30 minutes. Temperature variations were due to oven disparities. Untreated logs were kept in 66-liter clear plastic tubs in a screen house during the heat treatments.

Adult *P. juglandis* used in the re-infestation studies were reared from logs placed in emergence boxes stored in a screen house. On 24 July approximately 130 freshly emerged beetles were transferred directly onto each log in each individual tub. The tubs were sealed until 1 August when an additional 90 freshly emerged *P. juglandis* were transferred onto each log. On 5 August, logs used in re-infestation studies were brushed off to remove *P. juglandis* remaining on the log surface, and then placed in emergence boxes.

Cold Trial. Insects exposed to cold temperatures undergo physiological changes to avoid freeze injury (Bale 2002, Denlinger and Lee 1998), and lower lethal temperatures for *P. juglandis*

were determined by Luna and Sitz et al. (2013, submitted manuscript) under laboratory conditions. In this study, cold treatments were conducted using black walnut logs naturally infested with developing *P. juglandis*. The experiment included three treatments (untreated check, cold treatment, and cold treatment with subsequent beetle re-exposure) and 6 replications. Logs were sealed with Anchorseal on 23 July. Untreated logs were stored in a screen house while cold treatments occurred in a chest freezer set at -25°C. During exposure to cold treatments, logs were randomly placed in three Iron-Hold Contractor Bags® (Berry Plastics Group, Inc. Evansville, Indiana) where six of 12 log temperatures were recorded using data loggers and temperatures probes (see heat treatment). After the cold treatment, which ran for 6 days (31 July to 6 August), untreated and cold logs were boxed 6 August.

The logs designated for re-exposure with *P. juglandis* adults were placed in individual 66-liter clear plastic tubs. Approximately 200 newly emerged *P. juglandis* were placed directly onto each log on 6 August; three days later approximately 110 more freshly emerged *P. juglandis* were added to each log. *Pityophthorus juglandis* were brushed off and the logs were sealed 13 August.

Submergence Trial. Wood workers may use alcohol and water soaks to properly prepare logs before constructing products (Hamilton 2000). The efficacy of alcohol and water soaks were tested on *P. juglandis* infected material by completely submerging logs under either alcohol or water. There were 6 replications and 3 treatments (alcohol soak, water soak, and control). Treated logs were submerged under either 70% ethanol or water containing 0.5% of the surfactant Dyne-Amic® (Helena Chemical Company, Collierville, TN), and held submerged under cinder blocks until 31 July (8 days). On 31 July, alcohol and water soaked logs were removed and left to evaporate until 1 August when boxed. Untreated logs were sealed with

Anchorseal on 23 July and stored until boxed on 1 August.

Chipping Trial. Mechanical control, in the form of chipping, has effectively decreased wood borer emergence from infested logs (McCullough et al. 2007, Wang et al. 2000). Branch sections averaging 6.4 cm in diameter were used to determine if chipping is a viable option to control emerging *P. juglandis*. For each replication a single infested limb naturally infested with *P. juglandis* was used, approximately 61 centimeters in length. These were cut in half to comprise the two treatments (chipped and control), and the experiment contained 11 replications. A chipper (BX Wallenstein, EMB MFG Inc. St. Clements, Ontario) chipped the logs on 28 July, and the treatments were boxed on 31 July. The average volume of the chipped material was 4.4 L, and the size of the chips ranged from approximately 4.5 cm in length to 0.5 cm in length (Figure 2.1).



Figure 2.1. The size of black walnut wood chips used in the chipping experiment (measured in centimeters).

Statistical Analysis. Data collected were numbers of insects recovered from the collecting tubes of emergence boxes. All summary statistics use total emergence numbers. To

correct for normality, when evaluating *P. juglandis* emergence total emergence values were natural log transformed (\ln) in the insecticide trial, and in all other analyses total emergence values were increased by one in the natural log transformation prior to analysis ($\ln + 1$) (SAS 2012, JMP Pro 10 Statistics). Mean estimates and confidence intervals were back-transformed.

When evaluating the *P. juglandis* data, a linear model analysis of variance (ANOVA), followed by Tukey's honestly significantly different (Tukey's HSD) test, were used to test the effects of each treatment on the number of beetles emerged in the insecticide study ($\alpha = 0.05$) (all Tukey's HSD adjusted p-values are $P < 0.05$) (SAS 2012, JMP Pro 10 Statistics). In the heat, cold, and alcohol studies Least Square Means (LSMeans) Student's t pair-wise tests were used to determine differences in the emergence between the control and treated logs. A Fisher's exact test was used to check for binomial (yes/no) emergence in the heat re-infestation, cold re-infestation, and water soak treatments. The treated log emergence numbers were compared to the respective emergence numbers from control logs. Comparisons between chipping and control treatments were analyzed using an unpaired *t*-test allowing for unequal variance.

Data were analyzed for the *Cryptolestes*, *Corticotomus*, and *Theocolax* adults, along with *Enoclerus* larvae when emergence numbers of these species were great enough (above 12 specimens in the experiment). *Cryptolestes* and *Enoclerus* emergence numbers were analyzed with a Tukey's HSD ($\alpha = 0.05$) (all Tukey's HSD adjusted p-values are $P < 0.05$) in the insecticide trial whereas *Theocolax* and *Corticotomus*, data were analyzed by a Fisher's exact test. A Fisher's exact test was used to analyze *Cryptolestes* emergence in the heat and cold trials. In the soaking experiment, Fisher's exact test was used to compare *Cryptolestes* emergence from the control and the alcohol treatment while LSMeans was used to compare the control to the

water soak. In the chipping experiment, an unpaired t-test to allow for unequal variance used to analyze the *Cryptolestes* and *Theocolax* emergence data.

RESULTS AND DISCUSSION

Insecticide Trial. Insecticides decreased beetle emergence from the felled logs by at least 80%, but failed to completely control *P. juglandis* emergence (Table 2.1). Fewer beetles emerged from bifenthrin treated logs (32.5 ± 1.13) than from carbaryl treated logs (314.2 ± 1.14), and both insecticide treatments decreased emergence compared to control logs ($1,702.8 \pm 1.12$). *Pityophthorus juglandis* emerged from the carbaryl treated and control logs for the duration of the 15 week study, but stopped emerging from the bifenthrin treated logs after 12 weeks.

Greatest emergence occurred during the third collection week (Figure 2.2).

Table 2.1. The percent of black walnut logs from which *Pityophthorus juglandis* emerged after insecticide treatments were applied as a surface drenching spray to whole logs, the total number of beetles that emerged from each treatment, and the percent of beetle emergence after the treatment, relative to the control.

Treatment	<i>n</i> ^a	Infested Logs After Treatment (%) ^b	Total Beetles Emerged	Percent Emerged Relative to the Control ^c
Water check	10	100	21,512	.
Bifenthrin	10	100	485	2
Carbaryl	10	100	4,138	19

^a Number of logs tested in each treatment.

^b The percent of logs with beetle emergence after treatments were applied to sanitize them of *P. juglandis*.

^c The percent emerged relative to the control was determined by dividing the total number of beetles emerged from treated logs by the total number of beetles emerged from the control logs, and multiplying by 100.

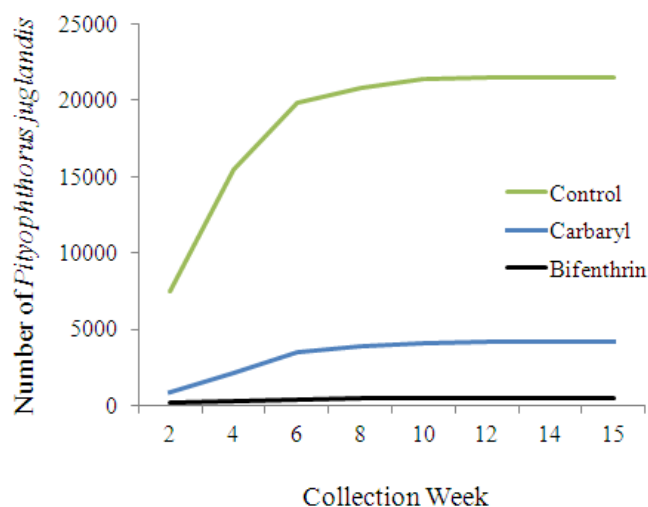


Figure 2.2. The accumulated bi-weekly emergence of *Pityophthorus juglandis* from water check walnut logs (control), from logs drenched with the insecticide carbaryl, or from logs drenched with the insecticide bifenthrin. Emergence was recorded from 30 July 2012 to 5 November 2012.

Previous studies found that laboratory bioassays using similar rates of the insecticides bifenthrin and carbaryl are capable of producing complete beetle mortality within 48 hours (Utley 2013). However, the use of these insecticides as a bark drench did not provide the ability to completely prevent beetle emergence. These results suggest insecticides can provide only limited ability to reduce the vectors of thousand cankers disease from emerging from felled logs or standing trees that harbor active beetle populations. Similar control has been documented using lindane (Lyon and Swain 1968, Osburn 1962). After the application of lindane, Lyon and Swain (1968) recorded a decrease in western pine beetle (*Dendroctonus brevicomis* LeConte) emergence ranging from 87 to 99 percent.

Heat Trial. Significantly fewer *P. juglandis* emerged from heated logs when compared to the control ($P < 0.0001$). Exposure of logs to air temperatures above 60°C for 30 minutes successfully killed all *P. juglandis* in thousand cankers diseased walnut logs (Table 2.2). This suggests that heat treatments can be an effective phytosanitary method for control of this species,

which is consistent with previous research on heat treatments of walnut slabs and laboratory bioassays with *P. juglandis* (Costanzo 2012, Luna and Sitz et al. 2013 submitted manuscript).

The temperatures and time durations needed to effectively disinfest wood of insects can vary. Nzokou et al. (2008) documented kiln temperatures of 65°C for 30 minutes while Myers et al. (2009) recorded temperatures of 60°C for 60 minutes were needed to sanitize infested ash logs of the emerald ash borer *Agrilus planipennis* (Fairmaire). In another study, Mushrow et al. (2004) reported control of the brown spruce longhorn beetle, *Tetropium fuscum* Fabricius, after exposing logs to 50°C for 30 minutes or 55°C for 15 minutes. In a similar study, Myers and Bailey 2011 followed ISPM 15 guidelines and heated logs to 56°C for 30 minutes to successfully sanitize logs of the Asian longhorned beetle, *Anoplophora glabripennis* Motschulsky. It should be noted that *P. juglandis* is restricted to cambium, unlike the above mentioned pests, and thus may be more easily killed by whole log heat treatments.

Although heat treatments eliminated initial *P. juglandis* populations some establishment was observed when heat treated logs were subsequently exposed to beetles. Eighty percent of the logs re-exposed to beetles were re-colonized with an average (\pm standard error) of 12.2 ± 9.88 beetles emerging from the re-exposed logs. The greatest *P. juglandis* emergence from the logs was eight to nine weeks after the treatment. This indicates that if felled walnut logs are re-exposed to *P. juglandis* after sanitation measures, they have the potential to harbor live beetles for extended periods.

Natural attack of heat treated logs by bark beetles, longhorned beetles, and weevils has been documented in recently milled green lumber (Petrice and Haack 2009). Beetles could colonize logs in order to feed, and/or reproduce. The greatest rate of *P. juglandis* emergence from the control logs was during the first collection week which occurred in the beginning of

August. In contrast, the greatest emergence from the logs re-infested with beetles occurred on weeks eight and nine (Figure 2.3), which is within the generation time of this insect suggesting successful reproduction in heat treated wood (Amman 1974). It is unknown whether or not *P. juglandis* retain reproductive capabilities in heat treated phloem tissue because beetle emergence did not surpass the number of beetles provided in the re-infestation experiment and logs were not destructively sampled. Brittle cambium, as seen in the heat treated logs, indicates low moisture content which is a possible limiting factor in reproduction and further sustainability of *P. juglandis*.

Table 2.2. The percent of black walnut logs with beetle infestation after heat treatments were applied to sanitize logs of *Pityophthorus juglandis*, the total number of beetles emerged from each treatment, and the percent of beetle emergence after the treatment, relative to the control.

Treatment	<i>n</i> ^a	Infested Logs After Treatment (%) ^b	Total Beetles Emerged	Percent Emerged Relative to the Control ^c
Control	10	100	1,764	.
Heat	10	0	0	0

^a Number of logs tested in each treatment.

^b The percent of logs with beetle emergence after treatments were applied to sanitize them of *P. juglandis*.

^c The percent emerged relative to the control was determined by dividing the total number of beetles emerged from treated logs by the total number of beetles emerged from the control logs, and multiplying by 100.

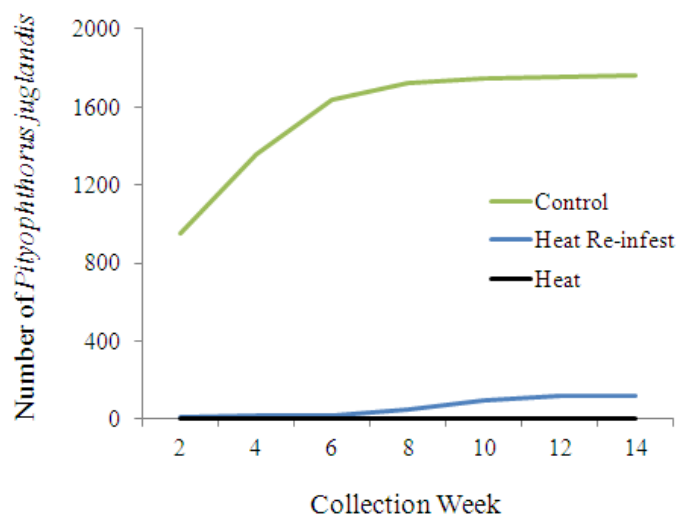


Figure 2.3. The accumulated bi-weekly emergence of *Pityophthorus juglandis* from untreated, or control, walnut logs, from logs after heat treatments were used to kill *P. juglandis*, or from logs heat treated and subsequently re-exposed to *P. juglandis*. Emergence was recorded from 6 August 2012 to 5 November 2012.

Cold Trial. Significantly fewer beetles emerged from the cold treated logs when compared to the control logs ($P = 0.0011$). Log cambium temperatures around -25°C sustained for seven days successfully sanitized felled logs of *P. juglandis* (Table 2.3). Lower lethal temperature studies for *P. juglandis* documented temperatures of -23°C and -25°C as 99 percent lethal (LT_{99}) to adult and larva populations respectively (Luna and Sitz 2013 submitted manuscript). The occurrence of lower lethal temperatures can impact the geographic distribution patterns of wood boring beetles (Ungerer et al. 1999) and can be used in pest management.

Pityophthorus juglandis emerged from the control logs and the cold treated logs with subsequent re-infestation. In both treatments, the highest numbers of *P. juglandis* emerging occurred during the first few weeks of the experiment (mid-August) (Figure 2.4). The mean number of *P. juglandis* emerged from the control logs and the cold treated logs with subsequent re-exposure are 187.7 ± 1.19 and 10.1 ± 1.08 respectively ($P = 0.0004$; $\text{df} = 1$).

As seen in the heat treatments, *P. juglandis* re-colonized walnut logs after the cold treatment. These results suggest that if cold treated logs are not stored properly, they may be suitable for sustaining *P. juglandis*. Beetles inhabited all of the logs after the cold treatment, and a similar number of beetles (mean \pm SE 11.0 ± 2.07) re-colonized the cold treated logs when compared to the heat treated logs (mean \pm SE 12.2 ± 9.88). However, fifty five percent of the beetles emerged from cold treated logs during the first week of the experiment, and emergence ceased after five weeks (Figure 2.4). This suggests that a brood did not develop within the cold treated logs.

Table 2.3. The percent of black walnut logs with beetle infestation after cold treatments were applied to sanitize logs of *Pityophthorus juglandis*, the total number of beetles emerged from each treatment, and the percent of beetle emergence after the treatment, relative to the control.

Treatment	<i>n</i> ^a	Infested Logs After Treatment (%) ^b	Total Beetles Emerged	Percent Emerged Relative to the Control ^c
Control	6	100	1,481	.
Cold	6	0	0	0

^a Number of logs tested in each treatment.

^b The percent of logs with beetle emergence after treatments were applied to sanitize them of *P. juglandis*.

^c The percent emerged relative to the control was determined by dividing the total number of beetles emerged from treated logs by the total number of beetles emerged from the control logs, and multiplying by 100.

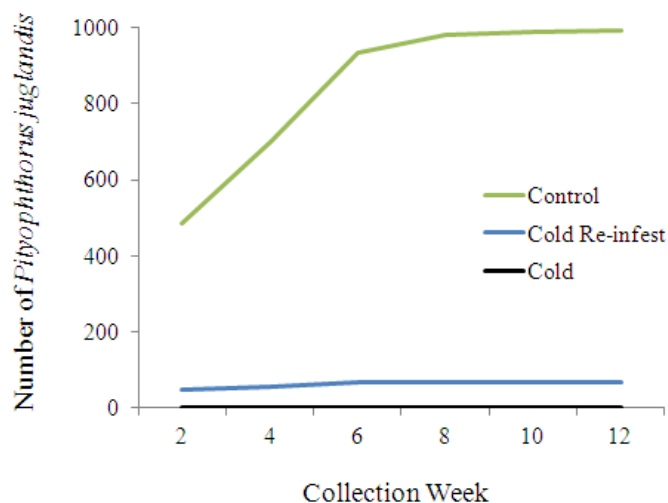


Figure 2.4. The accumulated bi-weekly emergence of *Pityophthorus juglandis* from untreated walnut logs (control), from logs after cold treatments were used to kill *P. juglandis* (cold) or from logs cold treated and subsequently exposed to *P. juglandis* (cold re-infest). Emergence was recorded from 20 August 2012 to 5 November 2012.

Submergence Trial. Beetle emergence was lower in alcohol soaked logs when compared to control logs ($P = 0.0303$). The least square means for the control (6.2 ± 1.40) and water soak (4.3 ± 1.38 ; $P = 0.686$; $df = 1$) treatments were not significantly different. Although *P. juglandis* emergence was similar in the two treatments, beetles emerged from only half as many water soaked logs as the untreated logs (Table 2.4). It is unclear why the water treatments produced inconsistencies. Furthermore, *P. juglandis* emergence greatly increased four to eight weeks after the water soak, but in the control logs emergence was highest in weeks one through three (Figure 2.5). Soaking logs in 70% alcohol for eight days provided an effective treatment for *P. juglandis* whereas soaking logs in water was an ineffective sanitation measure (Table 2.4).

Table 2.4. The percent of black walnut logs with beetle infestation after soaking treatments were applied to sanitize logs of *Pityophthorus juglandis*, the total number of beetles emerged from each treatment, and the percent of beetle emergence after the treatment relative to the control.

Treatment	<i>n</i> ^a	Infested Logs After Treatment (%) ^b	Total Beetles Emerged	Percent Emerged Relative to the Control ^c
Control	6	67	117	.
Alcohol Soak	6	0	0	0
Water Soak	6	33	115	98

^a Number of logs tested in each treatment.

^b The percent of logs with beetle emergence after treatments were applied to sanitize them of *P. juglandis*.

^c The percent emerged relative to the control was determined by dividing the total number of beetles emerged from treated logs by the total number of beetles emerged from the control logs, and multiplying by 100.

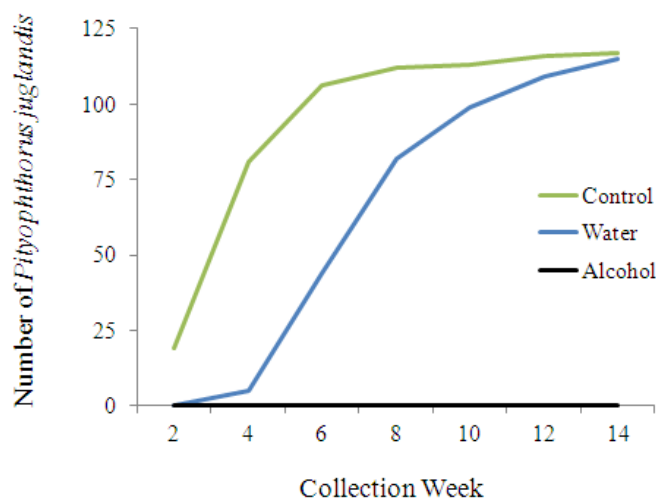


Figure 2.5. The accumulated bi-weekly emergence of *Pityophthorus juglandis* from untreated walnut logs, and from logs submerged for eight days under water containing 0.5% of the surfactant Dyne-Amic or 70% ethanol to determine if submergence treatments kill *P. juglandis*. Emergence was recorded from 13 August 2012 to 5 November 2012.

Chipping Trial. Chipping logs had a statistically significant ($P = 0.070$) impact on beetle emergence, with 94 percent of the *P. juglandis* killed during the chipping process (Table 2.5). Chipping infested material has been used to sanitize green ash *Fraxinus pennsylvanica* Marshall of emerald ash borer (McCullough et al. 2007) and silver maple, *Acer saccharum* Marshall, of Asian longhorned beetle (Wang et al. 2000). *Pityophthorus juglandis* emergence

ceased two weeks after the chipping treatment whereas beetles emerged from control logs for up to nine weeks (Figure 2.6). Chipping is a potential sanitation measure for *P. juglandis* infested material if it is properly taken care of during the two-week emergence period. Chipping infested logs fits management programs designed for material too small for milling.

Table 2.5. The percent of black walnut logs with beetle infestation after chipping treatments were applied to sanitize logs of *Pityophthorus juglandis*, the total number of beetles emerged from each treatment, and the percent of beetle emergence after the treatment, relative to the control.

Treatment	<i>n</i> ^a	Infested Logs After Treatment (%) ^b	Total Beetles Emerged	Percent Emerged Relative to the Control ^c
Control	11	91	867	.
Chipped	11	91	54	6

^a Number of logs tested in each treatment.

^b The percent of logs with beetle emergence after treatments were applied to sanitize them of *P. juglandis*.

^c The percent emerged relative to the control was determined by dividing the total number of beetles emerged from treated logs by the total number of beetles emerged from the control logs, and multiplying by 100.

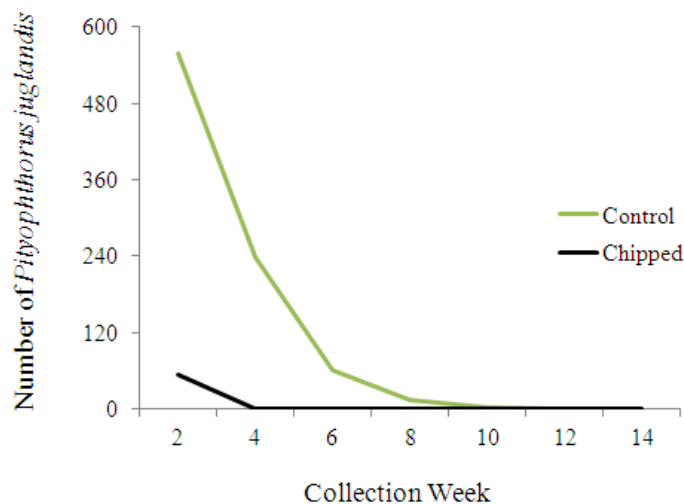


Figure 2.6. Bi-weekly emergence of *Pityophthorus juglandis* from combined control logs and chipped material. Emergence was recorded from 6 August 2012 until 5 November 2012.

Treatment Effects on Non-target Species. Individual trees support an entire community of organisms (Southwood et al. 1982). Gandhi and Herms (2010) estimate the

association of 282 arthropod species with ash (*Fraxinus* spp.), and Southwood et al. (2005) documented over 50 individual insect species in one square meter of oak (*Quercus* spp.). Furthermore, bark beetles are associated with many different organisms (Dahlsten 1982). For example, Dahlsten and Stephen (1974) documented over sixty species associated with *Dendroctonus ponderosae*.

Black walnut logs similarly host a wide range of arthropods (Appendix I). The insects associated with *P. juglandis* infested walnut material were of particular interest if they had predatory feeding habits or emerged from *J. nigra* logs in high abundance. *Cryptolestes* sp. (Coleoptera: Latridiidae), a fungivore, was the most abundant non-target beetle in the felled walnut logs (Appendix I, Table A.1.1). Similar to saproxylic species found in deciduous trees (Jonsell 2008), the *Cryptolestes* sp. associated with *J. nigra* lives under bark and feeds on detritus or fungus (Denux and Zagatti 2010).

Three predatory specimens of interest were *Enoclerus* sp. (Coleoptera: Cleridae), and *Corticotomus cavipes* (Fall) (Coleoptera: Trogossitidae), along with an undescribed parasitoid, *Theocolax* sp. (Hymenoptera: Pteromalidae) (Appendix I, Table A.1.1). Predatory beetles are known to appear closely after bark beetle infestations, and feed upon many different organisms (Dahlsten 1982). *Enoclerus* larvae emerged from the walnut logs, and both larvae and adults search prey galleries and feed upon bark beetles (Berryman 2012, Doane et al. 1936). *Corticotomus cavipes* are known to enter beetle galleries and prey upon *Pityophthorus* spp. (Doane et al. 1936). Parasitoid densities are usually small and do not tend to reflect bark beetle densities. The family Pteromalidae, which includes *Theocolax* spp., commonly parasitizes larvae and pupae of bark beetles (Dahlsten 1982). Adults oviposit through the bark, and therefore the eggs are not directly placed inside the bark beetle host. The larvae search for prey and are

ectoparasites once they find a host (Dahlsten 1982).

Emergence numbers from combined control logs in the heat, cold, and submergence experiments rendered at least seven times more *P. juglandis* than *Cryptolestes* sp. (Figure 2.7). At least 100 times more *P. juglandis* emerged than did *Corticotomus cavipes*, *Enoclerus* sp., and *Theocolax* sp. (Figure 2.8).

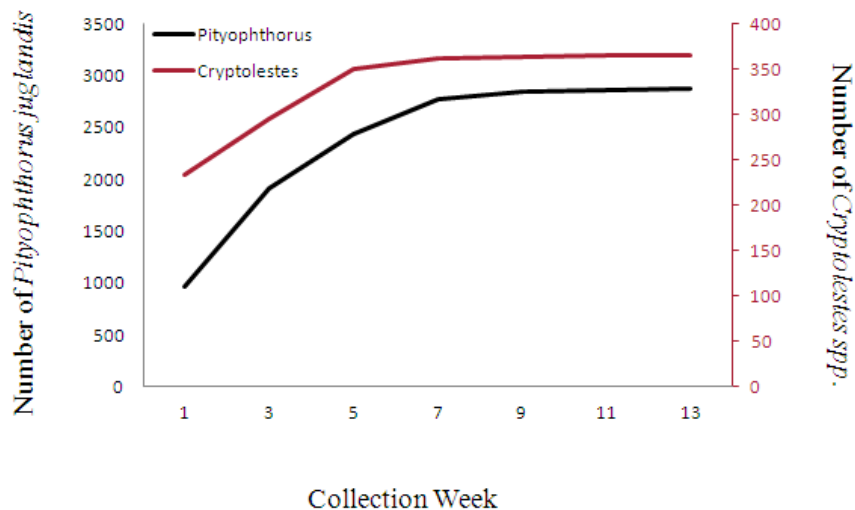


Figure 2.7. Accumulated emergence of *Pityophthorus juglandis* and *Cryptolestes* sp. from control walnut logs in the heat, cold, and submergence experiments. Emergence was tracked from 30 July 2012 to 5 November 2012 from all available logs. *Pityophthorus juglandis* is shown in black, and corresponds to the left hand legend. The colored right hand legend, shown in red, corresponds to *Cryptolestes* sp. emergence numbers. Emergence was quantified from all available logs between 30 July 2012 and 5 November 2012.

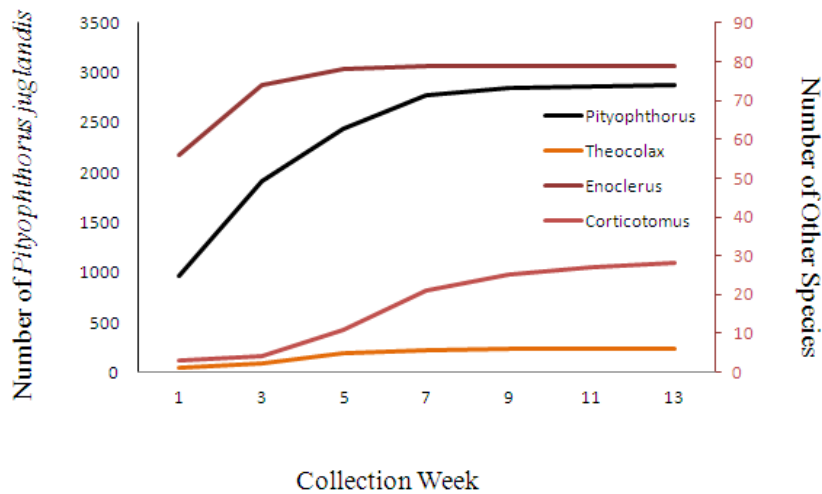


Figure 2.8. Accumulated emergence of *Pityophthorus juglandis* and the relatively abundant insects *Theocolax* sp., *Corticotomus cavipes*, and *Enoclerus* sp. from combined control logs from heat, cold, and soaking experiments. *Cryptolestes* sp. is shown in black, and corresponds to the black left hand legend. The right hand legend, shown in red, corresponds to *Theocolax* sp., *Corticotomus cavipes*, and *Enoclerus* sp. emergence numbers. Emergence was quantified from all available logs between 30 July 2012 and 5 November 2012.

Insecticide treatments had varying effects on the emergence of insects associated with walnut logs. Bifenthrin and carbaryl treatments decreased *Theocolax* sp. emergence by 100 percent ($P = 0.0108$). Fewer *Cryptolestes* sp. emerged from bifenthrin treated logs (0.7 ± 1.08) when compared to carbaryl treated logs (8.2 ± 1.19) and control logs (8.5 ± 1.21) (Table 2.6). Insecticide treatments did not have a statistically significant effect on *Enoclerus* sp. larvae as seen by similar emergence numbers from bifenthrin treated logs (0.2 ± 0.11), carbaryl treated logs (0.3 ± 0.11), and control logs (0.5 ± 0.20). *Corticotomus cavipes* emergence decreased from logs treated with bifenthrin by 100 percent when compared to the control ($P = 0.01$), and treating logs with carbaryl reduced emergence by 92 percent ($P = 0.06$). Researchers have documented negative effects on non-target predators, parasitoids, and associated species after control measures were performed to control bark beetles (Coster and Ragenovich 1976; Williamson and

Vite 1971). Similarly, *Theocolax* sp. and *C. cavipes* emergence decreased in response to one or both insecticide treatments intended for *P. juglandis*.

Table 2.6. The percent of black walnut logs with *Cryptolestes* sp. (Coleoptera: Latridiidae) after insecticide treatments were applied to sanitize logs of *Pityophthorus juglandis*, the total number of beetles emerged from each treatment, and the percent of beetle emergence after the treatment, relative to the control.

Treatment	<i>n</i> ^a	Infested Logs After Treatment (%) ^b	Total Beetles Emerged	Percent Emerged Relative to the Control ^c
Control	10	90	194	.
Bifenthrin	10	50	10	5
Carbaryl	10	100	172	89

^a Number of logs tested in each treatment.

^b The percent of logs with beetle emergence after treatments were applied to sanitize them of *P. juglandis*.

^c The percent emerged relative to the control was determined by dividing the total number of beetles emerged from treated logs by the total number of beetles emerged from the control logs, and multiplying by 100.

Fewer *Cryptolestes* sp. emerged from heat treated logs when compared to the control ($P = 0.0015$). Similarly, cold treatments intended to kill *P. juglandis* decreased emergence of non-target insects associated with black walnut. Fewer *Cryptolestes* sp. emerged from the cold logs ($P = 0.0076$) when compared to the control logs. One *Cryptolestes* sp. specimen emerged from the cold treated logs; out of the five tracked species it is the only species that survived the treatment.

Submerging walnut logs in water or ethanol had varying effects on *Cryptolestes* sp. emergence. *Cryptolestes* sp. emergence from the control logs was higher than emergence from the water soak treatment (11.5 ± 1.53 and 1.1 ± 0.05 respectively, $P < 0.0001$, $df = 1$). Submerging logs in 70% ethanol killed all *Cryptolestes* sp., and therefore a difference in emergence was detected between the control and ethanol treatments ($P = 0.0011$).

Chipping *J. nigra* logs not only greatly affected *P. juglandis* survival, but also decreased emergence of non-target insects. *Cryptolestes* sp. emergence was significantly decreased by the chipping treatment ($P = 0.0080$) (Table 2.7). *Theocolax* sp. emergence was similar ($P = 0.2647$) in both control logs and chipped material. However, forty-two wasps emerged from the control logs, but no wasps emerged from chipped material (Table 2.8). Six *Corticotomus cavipes* adults emerged from the control logs whereas one emerged from the chipped material. Similarly seven *Enoclerus* sp. larvae emerged from the control logs, and no emergence was recorded from the chipped material.

Table 2.7. The weekly number of *Cryptolestes* sp. that emerged from control logs and chipped material after a chipping treatment was applied to control *Pityophthorus juglandis* emergence.

Week	1	2	3	4	5	6	7	8	9	10	11	12	13
Number of <i>Cryptolestes</i> sp. Emerged													
Control	6	19	20	27	27	22	11	2	1	0	0	0	0
Chipped	1	2	0	2	1	0	0	0	0	0	0	0	0

Table 2.8. The weekly number of *Theocolax* sp. that emerged from control logs and chipped material after a chipping treatment was applied to control *Pityophthorus juglandis* emergence.

Week	1	2	3	4	5	6	7	8	9	10	11	12	13
Number of <i>Theocolax</i> sp. Emerged													
Control	0	0	8	7	1	7	12	5	2	0	0	0	0
Chipped	0	0	0	0	0	0	0	0	0	0	0	0	0

Managing *P. juglandis* infested logs is critical in order to salvage valuable lumber and prevent the spread of thousand cankers disease. Educating homeowners about the foreseen consequences thousand cankers disease will have on the *J. nigra* industry is critical in stopping the movement of infested material. Controlling beetle populations in salvaged city trees can help prevent disease spread to more communities and to the commercial walnut productions in the Eastern United States.

The results of these studies have implications for the management of *P. juglandis* infested wood collected from thousand cankers affected trees. Heat treatments of 60°C or greater for 30 minutes and cold treatments of -25°C for seven days effectively sanitize felled walnut logs of *P. juglandis*. Temperature treated logs remain potential hosts for *P. juglandis*, and therefore properly storing treated logs is necessary to sustain beetle-free material. Soaking logs in 70% ethanol for eight days is an effective management option for felled walnut logs, but water submersion is ineffective. Chipping can greatly decrease *P. juglandis* survival, but material must be properly managed for the two weeks during which limited emergence occurs. Finally, the insecticides bifenthrin and carbaryl can provide partial suppression of beetle emergence, but do not completely sanitize felled logs. All of these treatments can also affect other insect species associated with walnut wood.

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CHAPTER III. Fungicide Applications to Disinfest *Pityophthorus juglandis* (Coleoptera: Curculionidae) of *Geosmithia morbida*

INTRODUCTION

Thousand cankers disease of black walnut trees (*Juglans nigra*) is caused by the combined activity of two species: the walnut twig beetle, *Pityophthorus juglandis* (Blackman) and the filamentous ascomycete *Geosmithia morbida* (Tisserat et al. 2009, Kolarik et al. 2011). *Pityophthorus juglandis* causes minor damage to Arizona walnut (*Juglans major*) in its native range of New Mexico and Arizona (Blackman 1928, Cranshaw 2011). Recently, the beetle, when vectoring *Geosmithia morbida*, has emerged as a lethal pest of black walnut (*Juglans nigra*) (Tisserat et al. 2009), a species native to the eastern United States and highly valued for its timber and nuts (Harlow and Harrar 1969, Newton et al. 2009). The beetle creates galleries in the phloem and carries *G. morbida*, a canker-inducing fungus. Cankers form near beetle wounds and develop from numerous point introductions of the fungus, which cumulatively compromise the phloem (Tisserat et al. 2011). Eventual symptoms of affected trees include flagging, thinning, dieback of the upper canopy, often and ultimately followed by tree mortality (Tisserat et al. 2009).

The associations between bark beetles and their symbionts are often mutualistic (Six and Paine 1998, Paine et al. 1997). Fungi can increase the nutritional quality of the food source, decrease the amount of non-associated fungi in the environment, contribute to chemical communication, and increase reproductive success of bark beetles (Cardoza et al. 2006, Paine et al. 1997, Six and Paine 1998, Klepzig and Six 2004).

There are some suggestions that a mutualistic relationship also exists between the causal agents of thousand cankers disease, *P. juglandis* and *G. morbida*. *Geosmithia* spp. are vector-specific and found in association with all stages of the insect (Kolarik et al. 2004, Kolarik et al. 2005, Kolarik et al. 2008, Kubatova et al. 2004). Peachey (2012) documented a preference in *P. juglandis* larvae toward media containing *G. morbida* colonies, although adult beetles showed no positive movement toward *G. morbida* volatiles. However, as the two organisms are always found together it has not been possible to determine the nature of any mutualistic relationships that may exist or to establish the relative contribution of the two species in producing thousand cankers disease.

Historically, researchers have used multiple techniques in attempt to sterilize bark beetles of their associated fungi. For example, trials using White's solution (1 g HgCl₂/L sterile H₂O) (Kopper et al. 2003, Barras 1972), Hyamine 10-X (2 g/L sterile H₂O), mercuric chloride (1 g/L sterile H₂O) (Barras 1972), and 70% ethanol (Biedermann et al. 2009) were performed to decrease the amount of fungal colonies associated with beetles. Effective fungicides have not been tested to sterilize *P. juglandis* or *G. morbida*.

Fungicides are sometimes used to treat woody plants affected by fungi (Mueller et al. 2008; Amiri et al. 2008). Fungicides can be administered to trees by foliar applications (Mueller et al. 2008), or soil drenches (Appel and Kurdyia 1992), but trunk injections are commonly used for certain fungi that develop within the trunk and limbs, as does thousand cankers disease. For example, trunk injections of methyl 2-benzimidazole carbamate, along with multiple other fungicides were used for control of Dutch elm disease (Elliston and Walton 1979, Haugen and Stennes 1999). Trunk injections of propiconazole can be administered to *Prunus* spp. in attempt to control Armillaria root rot (Amiri et al. 2008). Additionally, propiconazole can prevent severe

crown loss in live oak and white oak trees infected with oak wilt (Appel and Kurdyia 1992, Eggers et al. 2005). Efficacy of fungicides against *G. morbida* has not been established, and would be the first step in determining if these products might be useful in thousand cankers disease management.

This research was conducted to determine the relative efficacy of three commonly used fungicide active ingredients in turf/ornamental pest management (azoxystrobin, propiconazole, and thiophanate-methyl) in a laboratory bioassay. In order to determine any mutualistic relationship between the two species, the association between the beetle and the fungus was explored in a series of studies using sterilizing agents in attempt to create fungus-free beetles.

MATERIALS AND METHODS

Fungicide Dilution Assays. The active ingredients used in the experiment represent a range of chemical classes (Mueller et al. 2008). Thiophanate-methyl (Cleary's 3336^F®, ClearyChemical, Dayton, NJ) a methyl benzimidazole carbamate, azoxystrobin (Heritage®, Syngenta Professional Products, Greensboro, NC) a quinine outside inhibitor, propiconazole (BannerMAXX®, Syngenta Professional Products, Greensboro, NC) a demethylation inhibitor, and azoxystrobin in conjunction with propiconazole (Headway®, Syngenta Professional Products, Greensboro, NC) were tested for their ability to kill *G. morbida*. The methods used were similar to Detweiler et al. (1983) and Golembiewski et al. (1995). Briefly, sterile technique was used to grow the *G. morbida* strain on ½-strength potato dextrose agar (PDA) amended with variable concentrations of the test fungicides. Fungicides were suspended in sterile water, and a stock solution of 1,000 µg/g of the active ingredient was made based on label concentrations. From the stock solution of fungicide, specific concentrations were added to autoclaved media via

a pipette (Table 3.1). Preliminary experiments were set up to determine the necessary concentrations of propiconazole and azoxystrobin high enough to inhibit *G. morbida* growth. Dilutions were created for azoxystrobin (Heritage), azoxystrobin and propiconazole (based on propiconazole label rates) (Headway), propiconazole (BannerMAXX), and thiophanate-methyl (Cleary's 3336^F). Each fungicide concentration contained 3 replicate plates.

Table 3.1. The increasing concentrations (µg/g) of the active ingredients (azoxystrobin, propiconazole, azoxystrobin in conjunction with propiconazole, and thiophanate-methyl) that were used in the serial dilution plating assay. *Geosmithia morbida* strain 1217 was plated onto ½-strength potato dextrose agar containing the specified amounts of active ingredients, and the subsequent growth was recorded.

Azoxystrobin ^a	Propiconazole ^b	Propiconazole and Azoxystrobin ^c	Thiophanate-methyl ^d
0.0 µg/g	0.0 µg/g	0.0 µg/g	0.0 µg/g
0.01 µg/g	0.01 µg/g	0.01 µg/g	0.01 µg/g
0.05 µg/g	0.05 µg/g	0.025 µg/g	0.05 µg/g
0.1 µg/g	0.1 µg/g	0.05 µg/g	0.1 µg/g
0.5 µg/g	0.5 µg/g	0.1 µg/g	0.5 µg/g
1.0 µg/g	1.0 µg/g	0.25 µg/g	1.0 µg/g
5.0 µg/g	5.0 µg/g	0.5 µg/g	5.0 µg/g
10 µg/g	10 µg/g	1.0 µg/g	10 µg/g

^a The active ingredient in the fungicide Heritage (0.5 lb azoxystrobin per gallon Heritage).

^b The active ingredient in the fungicide BannerMAXX (1.3 lbs propiconazole per gallon).

^c The active ingredients in the fungicide Headway (0.521 lb azoxystrobin per gallon and 0.868 lb propiconazole per gallon).

^d The active ingredient in the fungicide Cleary's 3336^F (4.0 lb thiophanate-methyl per gallon).

Geosmithia morbida strain 1217 was used for all dilution plating experiments; colonies were started from inoculum stored at -20°C. Strain 1217 was originally collected in October 2007 from a thousand cankers diseased *J. nigra* located in Boulder, Colorado. Plated colonies were grown on ½ PDA and incubated at 25°C under continuous light (VWR Batavia, IL). Similar to Blunt (2012), new mycelial growth was harvested for the dilution assay. To

standardize the inoculum size, a 5 mm plug was taken with a hollow punch. The plug was placed face down in the center of the experimental plates, and plates were stored in the incubator. *Geosmithia morbida* radial colony growth was measured in 48 to 72 hour increments. Measurements consisted of two diameters of the fungal colony; the first diameter was recorded at the widest point and the second measurement was taken perpendicular to the first. The fungal hyphae neared the edges of the plate after ten days, and therefore the experiments were terminated (Figure 3.1). If growth did not occur, colonies were re-plated on ½ PDA to determine if *G. morbida* was killed or inhibited. No re-growth occurred in colonies that were killed, whereas growth was documented in inhibited colonies.

In vitro, the effectiveness of fungicide active ingredients is determined by their ability to inhibit mycelial growth as measured by the concentration of active ingredient that is needed to reduce the fungal population by 50 percent (EC_{50}). The EC values are predicted using the relationship between the concentration of active ingredient and the relative growth of the fungus (Jo et al. 2006). The percent of active ingredient needed to inhibit or kill *G. morbida* was determined for all fungicide active ingredients used in the experiment, and the EC_{50}/EC_{90} values were calculated for azoxystrobin (Heritage), azoxystrobin and propiconazole (Headway), and propiconazole (BannerMAXX). To make these calculations, the concentration (lbs/gal) of active ingredient in the fungicide was converted to an amount of active ingredient ($\mu\text{g/g}$) that was determined to inhibit or kill *G. morbida*. Natural log transformations were performed on the average growth rate (mm/day) of *G. morbida* at each dilution concentration relative to the control. Transformed growth rate data were used to determine a dosage response curve along with the EC_{50} and EC_{90} values (Golembiewski et al. 1995). The dosage response curve was fit to a first order decay regression ($y = \theta_1 * \text{EXP}(-\theta_2 * (X)) + \theta_3$) (SAS 2012, JMP

Statistical Software, version 10). If theta 3 was below 0.5, it was fixed at zero. Using the regression equation, the effect of fungicide concentration on *G. morbida* mycelial growth rate was determined by back solving the predicted growth rate to obtain EC₅₀ and EC₉₀ values.

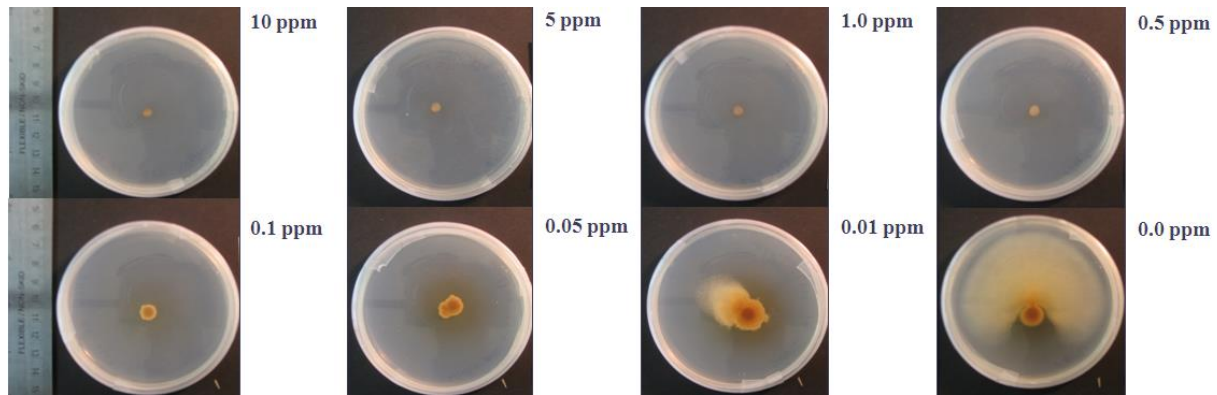


Figure 3.1. *Geosmithia morbida* growth on 1/2-strength potato dextrose agar amended with dilutions (ppm, parts per million or µg/g) of the active ingredient in the fungicide Banner Maxx.

Beetle Sterilization and *Geosmithia morbida* Isolation. Beetles were obtained from trees infected with thousand cankers disease, felled in Boulder or Fort Collins Colorado in 2012, and brought to the Colorado State University campus for use in rearing. *Pityophthorus juglandis* were extracted from the walnut logs and temporarily stored as groups in 1 mL tubes until the experiment began and each group was dumped into a 50 mL tube containing 20 mL of the treatment solution. Beetles were treated with deionized water as a control, 10% bleach, 70% ethanol, or the determined lethal concentration of the fungicide active ingredients in attempt to sanitize them of *G. morbida*. The treatment durations were modified from Kopper et al. (2003). After the specified time interval, the solution was dumped onto a screen where *P. juglandis* specimens were collected and transferred to the next tube with a sterilized paintbrush. Treated specimens were visually checked for survival immediately after the treatment, and stored at -18°C until maceration and fungal isolation. Beetles were macerated with a sterile mini-pestle, and then the solution was streaked onto 1/4 ++ PDA plates amended with 100 mg/liter

streptomycin sulfate and 100 mg/liter chloramphenicol (Tisserat 2009). Single spore fungal colonies were transferred to ½ PDA plates, and incubated until colony identification.

The first trial involved evaluations with azoxystrobin and propiconazole (Headway) a combination fungicide for sterilizing beetles of *G. morbida*. Four treatments were tested including: 10% bleach, 70% ethanol, 6 µg/g azoxystrobin in conjunction with 10 µg/g propiconazole (Headway) with 0.01% tween, and a water control. Ten adults or larvae *P. juglandis* were placed in sterile 50 mL tubes where they were systematically inverted in water for one minute, in each treatment solution for four minutes, and finally rinsed twice in water solutions for one minute each.

Propiconazole (Banner Maxx) was evaluated in a second trial to attempt beetle sterilization. Four treatments were tested including: 10% bleach, 70% ethanol, 10 µg/g propiconazole (BannerMAXX) with 0.01% tween, and a water control. Fifty adults were used in each treatment and three exposure durations were tested due to observed beetle mortality in the previous experiment; using treatment exposures 30 seconds, one minute, and two minutes. During exposures, the beetles were first immersed in water for one minute, treated for the test duration, and finally rinsed twice, each for one minute, in water.

RESULTS AND DISCUSSION

Fungicide Dilution Assays. Varying effects on *Geosmitha morbida* growth and survival were noted with the fungicide treatments. Propiconazole alone (Banner Maxx), or in combination with azoxystrobin (Headway), was most effective in inhibiting mycelial growth and killing the fungus when compared to other chemical classes. Propiconazole concentrations of 6.4 µg/g effectively kill *G. morbida* (BannerMAXX) (Figure 3.2).

Mycelial growth was inhibited by greater than 97 percent in potato dextrose agar amended with 10 µg/g or higher concentrations of the fungicide azoxystrobin (Heritage). However, regrowth of the fungus was noted immediately following the transfer of the original agar plug containing from the fungicide-amended to non-amended agar. Although azoxystrobin inhibits mycelial growth, 10 µg/g does not effectively kill *G. morbida* and the EC₉₀ for azoxystrobin was the highest of the effective fungicides in these tests (Figure 3.3). Less propiconazole is needed than azoxystrobin in order to inhibit mycelial growth by 90% (Table 3.2).

Geosmithia morbida was completely inhibited and killed in agar amended with 0.6 µg/g azoxystrobin and 1.0 µg/g propiconazole (Headway) (Figure 3.4). When the two active ingredients are used in combination, the effective concentration needed to decrease mycelial growth by 90% is 1,000 times less than if the active ingredients act alone (Table 3.2). Field studies are needed to determine if propiconazole alone or in conjunction with azoxystrobin will be effective fungicide treatments for standing trees with thousand cankers disease.

Thiophanate-methyl (Cleary's 3336^F) did not provide complete inhibition of *G. morbida* or fungal mortality at any test concentration (Figure 3.5). The relative growth rate of *G. morbida* was reduced by 20% with 10 µg/g thiophanate-methyl (Cleary's 3336^F), the highest test concentration. A regression line was not fit to the data due to insufficient *G. morbida* inhibition (Table 3.2, Figure 3.5).

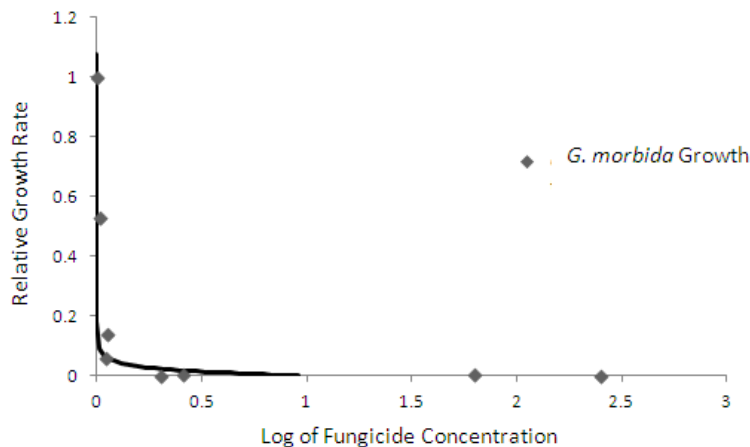


Figure 3.2. The mycelial growth rate of *Geosmithia morbida* strain 1217 on agar plates amended with increasing concentrations of propiconazole, the active ingredient in the fungicide Banner Maxx relative to the control. *Geosmithia morbida* measurements were taken after incubation at 25°C for 10 days. The first order decay regression line for *G. morbida* ($y = 0.98 * \text{EXP}(-51.34 * (X)) + 0$) is displayed.

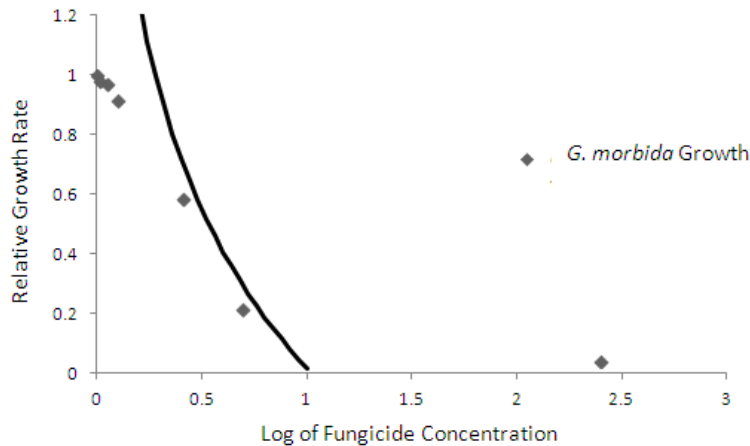


Figure 3.3. The mycelial growth rate of *Geosmithia morbida* strain 1217 on agar plates amended with increasing concentrations of azoxystrobin, the active ingredient in the fungicide Heritage relative to the control. *Geosmithia morbida* measurements were taken after incubation at 25°C for 10 days. The first order decay regression line for *G. morbida* ($y = 1.02 * \text{EXP}(-1.30 * (X)) + 0$) is displayed.

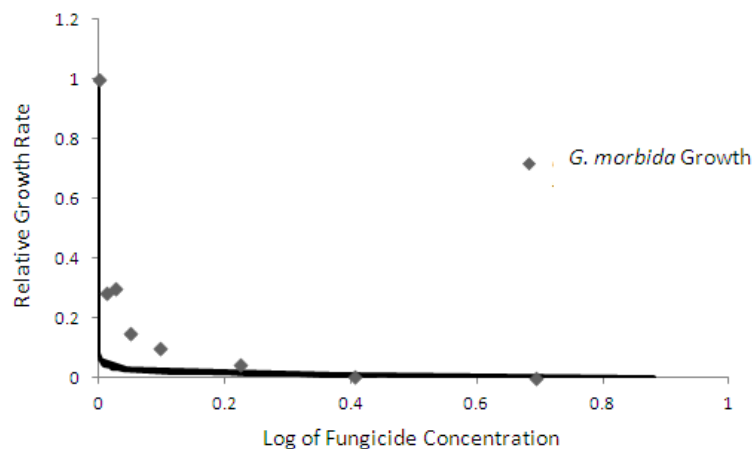


Figure 3.4. The mycelial growth rate of *Geosmithia morbida* strain 1217 on agar plates amended with increasing concentrations of the active ingredients azoxystrobin and propiconazole in the fungicide Headway relative to the control. *Geosmithia morbida* measurements were taken after incubation at 25°C for 10 days. The first order decay regression line for *G. morbida* ($y = 0.90 * \text{EXP}(-115.96 * (X)) + 0$) is displayed.

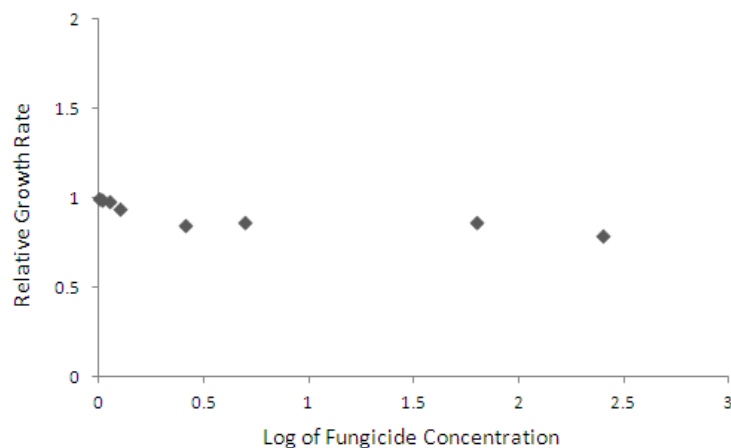


Figure 3.5. The mycelial growth rate of *Geosmithia morbida* strain 1217 on agar plates amended with increasing concentrations of thiophanate-methyl, the active ingredient in the fungicide Cleary's 3336^F relative to the control. *Geosmithia morbida* measurements were taken after incubation at 25°C for 10 days. The first order decay regression line for *G. morbida* is ($y = 0.16 * \text{EXP}(-4.27 * (X)) + 0.84$), but not displayed on the graph due to insufficient fungal inhibition.

The first order decay model for *G. morbida* growth at increasing concentrations of fungicide follows:

$$y = \text{theta } 1 * \text{EXP}(-\text{theta } 2 * (X)) + \text{theta } 3.$$

The estimated values for theta were calculated with a first order decay regression model fit to the relative growth data of *G. morbida* when exposed to azoxystrobin (Heritage), azoxystrobin and propiconazole (Headway), propiconazole (BannerMAXX), and thiophanate-methyl (Cleary's 3336^F) (Table 3.2).

Table 3.2. An analysis of *Geosmithia morbida* relative growth after the 1217 isolate was exposed to increasing concentrations of the active ingredients azoxystrobin, azoxystrobin in conjunction with propiconazole, propiconazole, and thiophanate-methyl. The first order decay response equation ($y = \text{theta } 1 * \text{EXP}(-\text{theta } 2 * (X)) + \text{theta } 3$) contains regression estimates for theta 1 and theta 2, which are used to calculate the effective concentration of fungicide active ingredient needed in the agar solution (μg active ingredient/g) to decrease mycelial growth of *G. morbida* by 50% or 90% (EC_{50} $y = 0.5$, and EC_{90} $y = 0.9$ respectively).

Fungicide Treatment	First Order Decay Regression	EC_{50} ($\mu\text{g/mL}$)	EC_{90} ($\mu\text{g/mL}$)
Propiconazole ^a	$y = 0.98 * \text{EXP}(-51.34 * (X)) + 0$	0.67	0.0016
Propiconazole and Azoxystrobin ^b	$y = 0.90 * \text{EXP}(-115.96 * (X)) + 0$	0.0051	0.0000033
Azoxystrobin ^c	$y = 1.02 * \text{EXP}(-1.30 * (X)) + 0$	0.55	0.096
Thiophanate-methyl ^d	$y = 0.16 * \text{EXP}(-4.27 * (X)) + 0.84$	- ^e	- ^e

^a The active ingredient in the fungicide BannerMAXX.

^b The active ingredients in the fungicide Headway.

^c The active ingredient in the fungicide Heritage.

^d The active ingredient in the fungicide Cleary's 3336^F.

^e Thiophanate-methyl did not inhibit mycelial growth enough to calculate EC_{50} or EC_{90} values, as shown by a theta 3 estimate above zero.

Beetle Sterilization and *Geosmithia morbida* Isolation. None of the treatments used to attempt sterilization of beetles of *G. morbida* were effective; plates streaked with macerated beetles produced *G. morbida* growth. Sterilization might have occurred on external portions of *P. juglandis*, but macerating the beetles exposed gut contents which could have contained viable

G. morbida. Similarly, after surface sterilization with ethanol, *Xyleborinus saxesenii* (Ratzeburg) were still able to produce fungal gardens (Biedermann et al. 2009). Modified White's solution reduced the incidence of *Ophiostoma* sp. fungi on *Ips pini* (Say), but complete fungal mortality was not documented (Kopper et al. 2003).

In contrast, surface sterilization of *Dendroctonus ponderosae* (Hopkins) and *D. jeffreyi* (Hopkins) pupae yielded sterile adults (Six and Paine 1998). In these species, newly emerged adults begin maturation feeding on the inside layer of their pupal chamber (Paine et al. 1997, Six and Paine 1998). Successful sterilization of *Dendroctonus* beetles may be attributed to the researchers' ability to intercept the beetles before adult maturation feeding occurred, which could have decreased the amount of fungal contaminants in the gut. *Pityophthorus juglandis* could exhibit maturation feeding on pupal chambers coated with *G. morbida*. Successful separation of these species may occur if pupae are sterilized and reared on *Geosmithia*-free diet. The association between *Geosmithia* spp. and their beetle vector lacks entomochory-related adaptations (i.e. mycangia) (Kolarik et al. 2008). Therefore the mechanism of fungal attachment may modify the means to successfully sterilize *P. juglandis* of the associated *G. morbida* conidia.

Even if the sterilization treatments were effective, many antifungal agents also kill insects (Barras 1972). *Pityophthorus juglandis* experienced high mortality submerged in fungicide or 70% ethanol at the shortest exposure time tested in these experiments, 30 seconds. In contrast, the 10% bleach and water check treatments did not kill the beetles. Kopper et al. (2003) documented live beetles after eight minutes of submersion in modified White's solution. Therefore, future attempts to sterilize *P. juglandis* could test higher concentrations of bleach or the modified White's solution.

In other beetle-fungal complexes the fitness of both species is enhanced. Six and Paine (1998) recorded lower fecundity within *Dendroctonus* populations reared in the absence of the symbiotic *Ophiostoma* fungi, and therefore concluded that beetle development is partially dependent on fungal communities. A greater amount of progeny was documented in *D. frontalis* Zimmerman carrying fungi when compared to sterile beetles (Barras 1973). Furthermore, *D. frontalis* exhibited higher lipid concentrations when associated with at least one fungal associate (Coppedge et al. 1995). The fitness of *P. juglandis* needs to be assessed in the presence of *G. morbida* and when sterilized of the fungus.

The active ingredient propiconazole, alone (Banner Maxx) or in combination with azoxystrobin (Headway), effectively inhibits and kills *G. morbida* populations. Neither azoxystrobin nor thiophanate-methyl (Cleary's 3336^F) provided complete inhibition of *G. morbida* or fungal mortality. *Pityophthorus juglandis* adults were not successfully sterilized using the fungicide active ingredients propiconazole (Banner Maxx) or azoxystrobin and propiconazole (Banner Maxx), 70% ethanol, or 10% bleach. Due to failure of successfully disassociating *P. juglandis* and *G. morbida*, it is unclear if sterile beetles cause tree mortality without their fungal counterpart, and what effect sterilization has on their lifecycles.

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APPENDIX I. Ecologically Important Insects Associated with Black Walnut (*Juglans nigra*)

Logs

Table A.1.1. Ecologically important Arthropods associated with black walnut logs. Including information on the classification, ecological role, and total number specimens emerged. Data were taken from the control logs in each experiment.

Class	Order	Ecological Role	Total Emerged
Arachnida	Aranea	Predator	6
Arachnida	Pseudoscorpionida	Predator	3
Arachnida	Acari	Unknown	2
Diplopoda	Polyxenida	Detritivore	1
Entognatha	Collembola	Detritivore	1
Insecta	Multiple ^a	Multiple ^a	27,061

^a See Table A1.2 for a breakdown of the Orders and ecological roles.

Table A.1.2. Ecologically important insects found in black walnut logs. Including information on the classification, ecological role, and total number specimens emerged. Data were taken from the control logs in each experiment.

Order	Family	Genus	species	Ecological Role ^a	Total Emerged
Coleoptera	Cleridae	<i>Enoclerus</i>	Unknown	Predator	86
Coleoptera	Scolytinae	<i>Pityophthorus</i>	<i>juglandis</i>	Phytophagous ^b	25,262
Coleoptera	Scolytinae	<i>Xyleborinus</i>	<i>saxeseni</i>	Phytophagous ^c	25 ^d
Coleoptera	Latridiidae	<i>Cryptolestes</i>	Unknown	Fungivore	639
Coleoptera	Trogossitidae	<i>Corticotomus</i>	<i>cavipes</i>	Predator	34
Diptera	Scenopinidae	<i>Scenopinus</i>	<i>velutinus</i>	Predator/Nectar	3
Hymenoptera	Pteromalidae	<i>Theocolax</i>	Unknown	Parasitoid	284
Psocoptera	Psocidae	Unknown	Unknown	Detritivore	728

^a The ecological role refers to the larval and adult feeding habits. If two categories are listed, the larvae and adult have different food sources, and the larval food source is listed first.

^b *Pityophthorus juglandis* feeds on the cambium tissue of black walnut.

^c *Xyleborinus saxeseni* feeds on the heartwood of black walnut.

^d The total number of *X. saxeseni* emerged was calculated as a percentage of *P. juglandis* emergence.